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TNF-α enhances phenotypic and functional maturation of human epidermal Langerhans cells and induces IL-12 p40 and IP-10/CXCL-10 production

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Abstract Dendritic cells (DC) play a central role in immunity/tolerance decision, depending on their activation/maturation state. TNF-α is largely produced in the skin under inflammatory conditions. However, it still remains to be defined how TNF-α modulates the activation status of human LC, the most specialized DC controlling skin immunity. Here, we reported that fresh immature LC, highly purified from healthy human skin and exposed for two days to TNF-α under serum-free conditions, expressed up-regulated level of co-stimulatory molecules (CD40, CD54, CD86), maturation markers (CD83, DC-LAMP), CCR7 lymph node homing receptor, and down-regulated Langerin level, in a dose-dependent manner. This mature phenotype is closely associated with enhanced LC allostimulatory capacity. Furthermore, TNF-α significantly increased the number of viable LC and decreased their spontaneous apoptosis. More importantly, TNF-α induced LC to produce both IFN-gamma-inducible-protein IP-10/CXCL10, a Th1-attracting chemokine and IL-12 p40. Bioactive IL-12 p70 was never detected, even after additional CD40 stimulus. The results implicate LC as an effective target through which TNF-α may up- or down-regulate the inflammatory skin reactions.

Keywords: Human Langerhans cells; Cytokines; Maturation

1. Introduction

Dendritic cells (DC) represent a family of professional antigen-presenting cells that are found in most tissues [1,2]. Langerhans cells (LC) constitute a special subset of immature DC, localized in the basal and suprabasal layers of the epidermis. They are characterized by the lectin Langerin/CD207, an endocytic receptor that induces Birbeck granules formation [3]. LC play a key role in the initiation of cutaneous immune responses by capturing and processing foreign antigens and migrating to the regional lymph nodes where they present the processed antigen to naive T cells [1,2]. When leaving from the skin, LC down-regulate Birbeck granules, as well as anchor receptors, such as E-cadherin, which allows detachment from the surrounding keratinocytes or the basal lamina [1,2]. Simultaneously, chemokine receptors such as CCR7 are induced to guide the DC through the afferent lymph into the regional lymph nodes [4]. During this migration, immature LC lose antigen uptake and processing properties as they differentiate into mature cells specialized for T cell stimulation [1,2]. This process is referred to as DC maturation or activation. Fully mature DC are recognized by their strong surface expression of MHC class I/class II, CD54, CD80, CD86, CD40 co-stimulatory molecules, CD83 and DC-LAMP maturation markers, and by their ability to produce pro-inflammatory cytokines, such as bioactive IL-12 p70 [5].

Although LC migration/maturation are crucial biological events in the initiation of cutaneous immune responses, it is not yet clear whether they are coordinated or can be induced independently. However, maturation of peripheral DC represents a key control point in determining the outcome of immune response. Indeed, while fully mature DC induce immunity, immature DC, characterized by the lack of co-stimulatory molecules and maturation markers, not only fail to prime T cells effectively but also serve to promote tolerance [5,6]. Recently, a third developmental stage of DC maturation has been proposed: "semi-mature" DC are phenotypically mature but failed to secrete inflammatory cytokines [5].

It is well documented that mobilization of LC to lymph nodes is triggered by inflammatory mediators and especially TNF-α [7]. The cytokine is largely produced in the skin, mainly by keratinocytes, in response to various skin infection or injury [8]. Although TNF-α is a well-known maturation factor for human DC generated in vitro from cord blood, bone marrow progenitors, peripheral blood monocytes [2,5], its effect on the resident epidermal LC has been far less studied. In the murine system, Koch et al. [9] first reported that TNF-α maintains fresh LC in a viable, but functionally immature state, probably due to its inability to induce up-regulation of CD86 [10], crucial for effective T-cell stimulation. In this study, we applied a slight modification of a previously described technique [11] to isolate highly purified immature LC (>90%) from healthy human skin. We used these cells as an in vitro system, with

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Abbreviations: LC, Langerhans cells; MFI, mean fluorescence intensity; PI, propidium iodide

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negligible interference of keratinocyte-derived cytokines, under serum-free culture conditions, to analyze the activating potential of TNF-α on their phenotype, function, apoptosis and cytokine/chemokine secretion.

2. Materials and methods

2.1. Culture medium and cytokines

Culture medium was X-VIVO-15 supplemented with 1% gentamycin (Sigma, St. Louis, MO) and 200 U/ml recombinant human GM-CSF (specific activity: 2×10^6 U/mg, a generous gift from Schering-Plough Research Institute, Kenilworth, NJ), called, thereafter, complete medium. Recombinant human TNF-α (specific activity 2×10^7 U/mg) was from Roche Diagnostics (Basel, Switzerland), TGF-β1 (specific activity 50×10^6 U/mg) from R&D Systems Europe Ltd (Abington, UK) and interferon-γ (specific activity 4×10^7 U/mg) from Amersham, Les Ullis, France. Human recombinant IL-10 (specific activity 2×10^7 U/mg) was kindly provided by Schering-Plough Research Institute.

2.2. Human epidermal Langerhans cell isolation and culture

LC were highly purified using a slightly modified, previously described technique [10]. Briefly, epidermal cell suspensions were obtained by trypsinization of human abdominal skin obtained by plastic surgery (0.05% trypsin, Difco Laboratories, Detroit, MI, 1 h at 37 °C). LC were enriched by three successive density gradient centrifugations on Lymphoprep (Flobio SA, Courbevoie, France). The resulting population routinely consists of >90% of LC, as assessed by flow cytometry using anti-HLA-DR-FITC and anti-Langerin-PE. Highly purified LC (1×10^6 cells/well) were cultured, or not, for 2 days in 6-well tissue culture plates (Costar Corp., Cambridge, MA) in complete medium, in the presence or not of TNF-α or IL-10 at different concentrations before carrying out phenotypic and functional assays.

2.3. CD40 ligand-mediated Langerhans cell maturation

Fibroblastic L cells, transfected with the human CD40 ligand (CD154c) and used for CD40 triggering on human epidermal LC, were kindly provided by Schering Plough Laboratories (Dardilly, France). L cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and irradiated at 80 Gy before use. 10^6 purified LC were seeded with 10^5 CD154c onto 24 well cultures plates, in X-VIVO-15 medium supplemented with 2% fetal calf serum (Myoclone, Gibco BRL Life Technol) and interferon-γ (500 U/ml). After two days, LC were recovered by flushing the plates with RPMI medium and viable cells were recovered after centrifugation on Lymphoprep before carrying out phenotypic and functional analysis.

2.4. Antibodies and flow cytometry

LC were washed in PBS containing 1% bovine serum albumin, and incubated for 30 min at 4 °C with a panel of FITC- or PE-labeled mouse mAbs at the appropriate concentration or with irrelevant isotype-matched mouse Igs at the same concentration. Cells were washed and, for indirect staining, further incubated for 30 min at 4 °C with FITC-conjugated $F(ab)'_2$ fragments of goat anti-mouse Ab. The following monoclonal antibodies were used: anti-HLA-DR-FITC (B8.12.2, IgG2b), anti-CD54-FITC (84H10, IgG1), anti-CD80-FITC (MAB 104, IgG1), anti-CD83 (HB15A, IgG2b), anti-CD40 (mAb89, IgG1), anti-Langerin-PE (DCGM4, IgG1), anti-DC-LAMP (104.G4, IgG1), all from Immunotech (Marseille, France); anti-CD86-FITC (2331FUN-1, IgG1), anti-CD86-PE (2331FUN-1, IgG1), from BD Pharmingen (San Diego, CA); anti-CD1a-FITC (NA1/34, IgG2a), anti-CD1a-PE (NA1/34, IgG2a), anti-CD14-FITC (TUK4, IgG2a) from Dako (Glostrup, Denmark); anti-CCR6-PE (53103.111, IgG2b) and anti-CCR7-FITC (150503, IgG2a), anti-TNF-R1-FITC (16803.1, IgG1) and anti-TNF-R2-FITC (22235.311, IgG2a) from R&D Systems Europe Ltd (Abington UK). For double labeling, cells were first stained with FITC-conjugated mAbs followed by PE-conjugated anti-HLA-DR. Intracellular staining for DC-LAMP were carried out by using the Intrastain Fixperm kit (Dako), according to the manufacturer's instructions. Analysis of fluorescence staining was performed with a FACScan flow cytometer (Becton Dickinson, Le Pont de Claix, France) using the CELLQuest Software. LC population was gated electronically by cell size and CD1a expression.

2.5. Quantification of apoptotic Langerhans cells

LC cultured in complete medium with or without different concentrations of cytokines were evaluated for apoptosis by double staining with FITC-labeled annexin V and PI according to the manufacturer's recommendations (Annexin V-FITC kit, Immunotech). Sample analysis was performed by flow cytometry, as described above.

2.6. T cell proliferation

Allogeneic T cells were isolated from peripheral blood, by rosetting with sheep red blood cells as previously described [12]. The T-cell population contained more than 95% $CD3^+$, as assessed by flow cytometry. Mixed lymphocyte reactions were carried out in 96-well round-bottomed plates by adding 105 allogeneic T cells to a graded number of LC. In some experiments, the cells were previously fixed (or not) with 1% paraformaldehyde for 30 min on ice. Triplicate cultures were maintained for 5 days at 37 °C in a humidified atmosphere of 5% CO_2. T cell proliferation was assessed at day 5 by addition of 1 μCi of [^{3}H]-methyl-thymidine per well (25 Ci/mmol, Amersham, Les Ulis, France) for the final 18 h of culture. Cells were collected on glass-fiber filter using a 96-well Harvester (Inotech, Switzerland), and thymidine incorporation was measured with a direct beta-counter (Matrix 96, Packard Instrument Co., Meriden, CT). Results were expressed as the mean cpm ± S.D. of triplicate cultures.

2.7. Cytokine production

Supernatants from cytokine treated or not-treated highly purified LC, as well as those of CD40L-activated LC were harvested after two days, and kept at −80 °C before use. The production of IL-1ß, IL-10 and IL-12 p70 was measured by ELISA using 96-well microtiter plates (R&D Systems Europe Ltd), according to the manufacturer's instructions: IL-10 (sensitivity: 7.80 pg/ml); IL-12 p70 (sensitivity: 0.625 pg/ml) and IL-1ß (sensitivity: 3.90 pg/ml). Several other cytokines were measured using BD Cytometric Bead Array (BD Biosciences, Mountain View, CA) and LUMINEX Beadlyte cytokine detection system (Upstate Biotechnology, Lake Placid, NY), according to the manufacturers' protocol. All supernatants were analyzed in duplicate and results were expressed as the mean amount of cytokine per ml (pg/ml ± S.D.).

2.8. Statistics

For statistical evaluation of significance, the Student's *t*-test was performed. Only *P*-values less than 0.05 were considered to be significant.

3. Results

3.1. TNF-α enhances phenotypic maturation of highly purified human epidermal LC

We first investigated the effect of TNF-α on the phenotypic maturation of highly purified human LC (>90% purity) after a 2-day incubation in serum-free medium. IL-10, which is known to inhibit human LC maturation [10], was used as a negative control. Fig. 1 illustrates the results of a representative experiment and Table 1 summarizes the results obtained from different donors. Freshly isolated LC revealed the typical features of immature DC, i.e. a lack of CD80, CCR7 and DC-LAMP; low levels of CD40, CD54, CD83 and CD86; and high levels of HLA-DR, Langerin, CD1a and CCR6. Upon a 2-day culture in serum-free medium, human LC up-regulated the expression of HLA-DR, CD40, CD54, CD83 and CD86, whereas CD1a and Langerin expression were decreased at the cell surface. Moreover, CD80, CCR7 and DC-LAMP were induced on epidermal LC, whereas CCR6 was lost.

As shown in Fig. 1 and Table 1, TNF-α clearly enhanced the expression of most activation and maturation markers in a dose-dependent way. Especially, CD40, CD54, CD83, CD86, CCR7 and DC-LAMP were significantly upregulated on LC, as compared to the cells incubated in medium alone

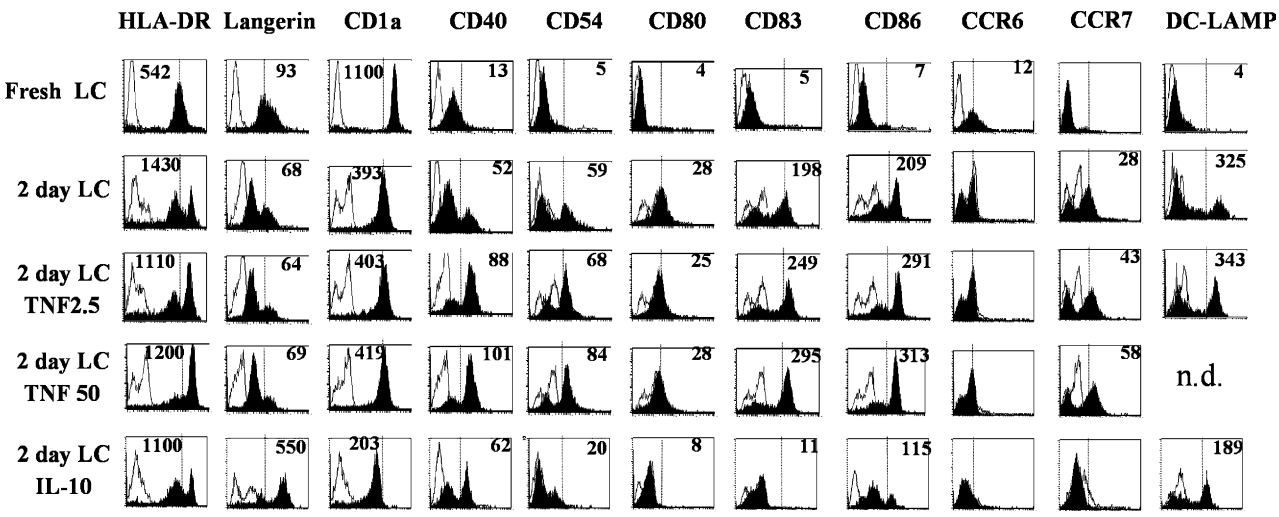

Fig. 1. TNF-α increases phenotypic maturation of human epidermal LC. Human LC were stained with a panel of FITC- or PE-conjugated mAbs immediately after their isolation from skin samples (fresh LC), or after a 2-day culture in serum-free medium (2 day LC), supplemented or not with TNF-α at 2.5 (2 day LC, TNF 2.5), 50 ng/ml (2 day LC, TNF 50), or IL-10 at 1.25 ng/ml (2 day LC IL-10). Filled histograms represent specific and open histograms isotype-matched control antibodies. Vertical lines correspond to the peak channel of fluorescence intensity of a given antigen on 2-day LC. The mean fluorescence intensity is reported in each histogram. n.d. not determined. Results are representative of six independent experiments.

($P < 0.01$). Not only was the intensity of the markers increased, but also the percentage of positive cells. By contrast, TNF-α significantly reduced cell surface Langerin expression. As expected, LC treatment with IL-10 strongly decreased the expression of CD40, CD54, CD80, CD86 and DC-LAMP and nearly abolished that of CD83 and CCR7 (Fig. 1, Table 1). Concurrently, Langerin expression was clearly upregulated at the cell surface ($P < 0.01$). Of note, by contrast with DC generated from $CD34^+$ progenitors [13], IL-10 did not maintain CCR6 expression. These results showed that TNF-α favors phenotypic maturation of freshly isolated human LC.

3.2. TNF-α enhances the capacity of human LC to induce primary allogeneic T cell response

We next determined whether up-regulation of co-stimulatory molecules and maturation markers on TNF-α treated LC might correlate with enhanced antigen-presenting function. Viable LC, either freshly isolated from skin samples, or recovered from a 2-day culture in the presence or not of TNF-α (2.5, 30 and 50 ng/ml) and directly used as antigen-presenting cells, exhibited similar capacity to stimulate allogeneic T cells (not shown). Since LC maturation is known to occur during the 6-day mixed LC-T cell reaction and may, therefore, alter the results, LC were fixed with paraformaldehyde prior to their addition to the T cells, as previously described [14]. As illustrated in Fig. 2, previous TNF-α exposure strongly enhanced the allostimulatory capacity of fixed LC, even at low doses (2.5 ng/ml). This effect paralleled increasing concentrations of the cytokine. Of note is the inability of fixed fresh LC, as compared with fixed-incubated LC, to induce T cell proliferation, as previously reported [14]. In agreement with previous data [11], LC incubation with IL-10 impaired human LC allostimulatory function in a dose dependent way (not shown).

3.3. TNF-α increases the relative number and the maturation level of a large-sized/HLA-DR^{high}/$Langerin^{low}$ LC subset

As shown in Figs. 1 and 3A, most freshly isolated immature LC homogeneously stained with anti HLA-DR mAb. By contrast, after a 2-day culture, LC comprised two subpopulations exhibiting either a low or a high HLA-DR fluorescence intensity. Addition of TNF-α significantly increased the relative number of HLA-DR^{high} LC ($P < 0.001$), with a concomitant decrease in the relative number of HLA-DR^{low} LC ($P < 0.001$), without altering their respective fluorescence intensity level (Fig. 3A). Thus, in 6 experiments, the mean percentage of HLA-DR^{high} and HLA-DR^{low} LC was 54.2 ± 8.2% and 45.8 ± 13.2% in the control LC suspension, whereas it averaged 71.1 ± 9.9% and 28.9 ± 10.1% in the TNF-α -treated (30 ng/ml) LC suspension, respectively. The two LC subsets could be easily distinguished by their FSC/SSC profiles, the HLA-DR^{high} subset exhibiting the largest size (Fig. 3A). Interestingly, Langerin expression was markedly lower in the HLA-DR^{high} than in the HLA-DR^{low} LC population (Fig. 3A).

We then compared expression of several markers and TNF receptors on the two LC subpopulations (Fig. 3B). Results showed that the small-sized/HLA-DR^{low} LC strongly expressed Langerin and CD1a but very low levels of most co-stimulatory molecules, maturation markers and TNF-R2. Furthermore, CCR6, CCR7 and TNF-R1 were never detected at the cell surface. Addition of TNF-α did not substantially alter the results. By contrast, the large-sized/HLA-DR^{high} LC expressed far lower level of Langerin and far higher levels of co-stimulatory molecules, maturation markers and TNF-R2 (Fig. 3B), while CCR6 and TNF-R1 (not shown) were undetectable. After treatment with TNF-α, the HLA-DR^{high} LC subset showed a clear up-regulation of CD40, CD54, CD83, CD86, CCR7 and DC-LAMP (Fig. 3B). The TNF-R2 expression remained unchanged. Collectively, these results show that TNF-α strongly enhances the relative number and the matura-

Table 1
TNF-α up-regulates expression of co-stimulatory molecules, maturation markers and CCR7, but down-regulates Langerin expression on human epidermal LC

	DR	Langerin	CD1a	CD40	CD54	CD80	CD83	CD86	DC-LAMP	CCR7
MFI of LC treated with TNF-α/MFI of untreated LC										
Expt #1	1.24	0.75	1.08	1.43	1.14	0.79	1.57	1.83	n.d.	1.26
Expt #2	1.15	0.76	1.13	2.53	2.12	0.89	1.34	2.15	1.27	1.94
Expt #3	1.20	0.42	1.18	1.33	1.72	n.d.	1.78	2.07	1.34	1.43
Expt #4	0.95	0.81	1.07	1.75	1.49	1.02	1.39	1.64	1.60	1.53
Expt #5	1.09	0.57	1.08	2.4	1.61	0.91	1.42	1.95	1.36	1.84
Expt #6	1.10	0.62	1.11	1.85	1.68	0.95	1.62	1.98	1.42	1.75
Mean ± S.D.	1.12[a] ± 0.10	0.66[a] ± 0.15	1.11[a] ± 0.04	1.88[a] ± 0.49	1.63[a] ± 0.32	0.91 ± 0.08	1.52[a] ± 0.17	1.94[a] ± 0.18	1.40[a] ± 0.12	1.63[a] ± 0.26
	DR	Langerin	CD1a	CD40	CD54	CD80	CD83	CD86	CD86	CCR7
MFI of LC treated with IL-10/MFI of untreated LC										
Expt #1	0.90	2.27	0.85	0.61	0.16	0.54	0.15	0.59	0.09	
Expt #2	0.97	2.17	0.83	0.43	0.21	n.d.	0.29	0.62	0.29	
Expt #3	1.06	2.07	0.98	0.52	0.32	n.d.	0.07	n.d.	0.11	
Expt #4	1.15	1.98	0.92	0.48	n.d.	0.42	n.d.	n.d.	n.d.	
Mean ± S.D.	1.02 ± 0.11	2.12[a] ± 0.13	0.90 ± 0.07	0.51[a] ± 0.08	0.23[a] ± 0.08	0.48 ± 0.08	0.17[a] ± 0.11	0.60 ± 0.02	0.16[a] ± 0.09	

Phenotypic analysis was carried out on purified epidermal LC cultured for 2 days in serum-free medium, with or without TNF-α (50 ng/ml) or IL-10 (1.25 ng/ml). LC were stained with a panel of FITC- or PE-conjugated mAbs and fluorescence was analyzed with a FACScan. Results for a given antigen are expressed as the following ratio: MFI in the presence of the cytokine/MFI in the absence of the cytokine.
[a]Value significantly different from that of control.

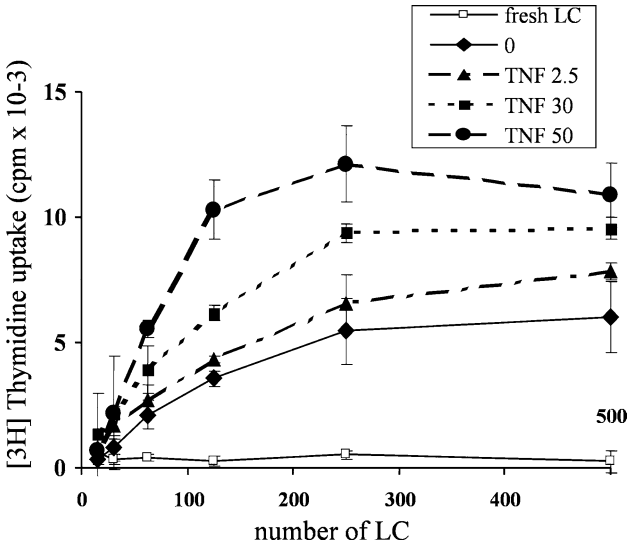

Fig. 2. TNF-α enhances the LC capacity to induce allogeneic T cell proliferation. Highly purified LC were fixed with paraformaldehyde either after their isolation from skin samples (fresh LC), or after 2-day culture in serum-free medium supplemented or not (0) with TNF-α at 2.5 (TNF 2.5), 30 (TNF 30) or 50 ng/ml (TNF 50). After extensive washing, LC were enumerated and graded numbers of viable cells were added to allogeneic T cells. After 5 days, T cell proliferation was assessed by addition of [^{3}H]-thymidine. Results are expressed as the mean cpm ± S.D. of triplicate cultures and are representative of 5 separate experiments. T cells alone gave less than 80 cpm.

tion stage of a large sized/HLA-DR^{high}/Langerin^{low} LC subpopulation.

3.4. TNF-α enhances human LC survival by inhibiting apoptosis of the HLA-DR^{high}/Langerin^{low} LC subset

In all the experiments, TNF-α treatment increased the number of recovered viable LC ($P < 0.001$). Indeed, in 6 experiments, the viable cell yield at day 2 averaged 66.9 ± 4.9% and 75.6 ± 9.1% in the presence of TNF-α at 2.5 and 50 ng/ml, respectively, whereas it was only 30.2 ± 7.1% in the absence of the cytokine. To test whether TNF-α might protect LC from spontaneous apoptosis, we compared the number of apoptotic cells in the two LC subpopulations, using annexin V-FITC staining. After a 2-day culture in serum-free medium, apoptotic cells were mostly localized in the HLA-DR^{low}/Langerin^{high} LC population (Fig. 4A). Interestingly, TNF-α decreased the number of apoptotic cells, in a dose-dependent way. The results were confirmed using double staining with annexin V-FITC/propidium iodide (Fig. 4B). As compared to the HLA-DR^{high}, the HLA-DR^{low} LC population mostly comprised cells in early and especially late apoptosis. In the presence of TNF-α, the percentage of viable cells was upregulated in a dose-dependent way, in the HLA-DR^{high}/Langerin^{low} LC subset.

3.5. TNF-α induces LC secretion of IP-10 and IL-12 p40

We next examined the effect of TNF-α on the capacity of LC to produce inflammatory cytokines and chemokines. CD40 ligation combined with T cell-derived signals, such as IFN-γ, known to induce maximal cytokines production by DC [15], was used as a positive control. As shown in Table 2, cultured LC spontaneously produced high levels of IL-8, minimal levels

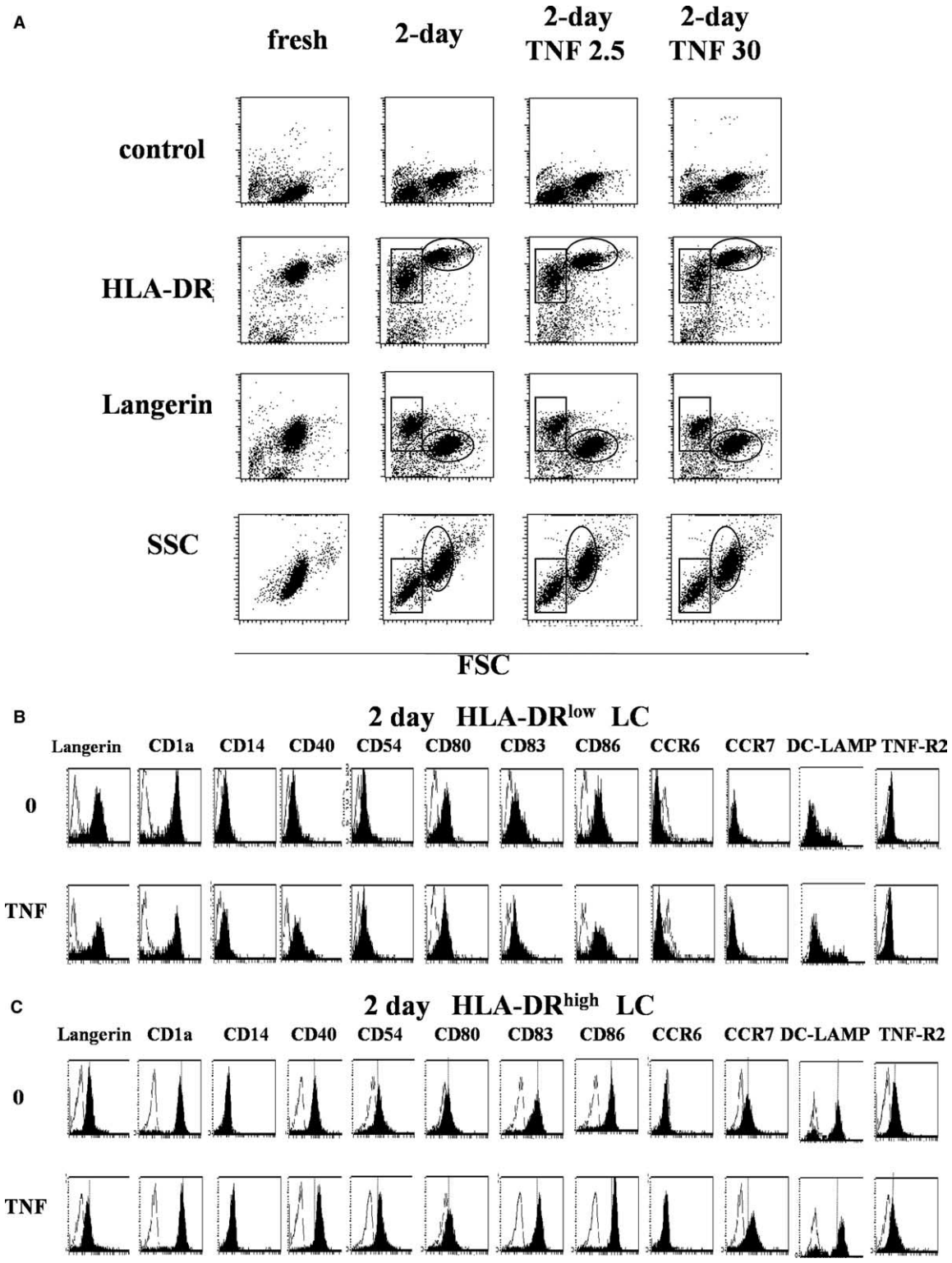

Fig. 3. TNF-α promotes the maturation of a high-sized/HLA-DR^{high}/Langerin^{low} LC subset. (A) Isolated human epidermal LC were stained with FITC-labeled anti-HLA-DR, anti-Langerin or isotype-matched control antibody, either immediately after their purification from skin samples (fresh) or a 2-day culture in serum-free medium supplemented or not (2 day) with TNF-α at 2.5 (2 day, TNF 2.5) or 30 ng/ml (2 day, TNF 30). Cells were analyzed without any gating. HLA-DR^{+} LC were subdivided into HLA-DR^{low} (rectangle) and HLA-DR^{high} (oval) subpopulations. (B) Each of the two HLA-DR subpopulations was gated electronically by cell size (FSC/SSC dot plots) and analyzed independently for expression of various surface antigens. Filled histograms represent specific and open histograms isotype-matched control antibodies. Data are representative of six experiments using skin from different donors.

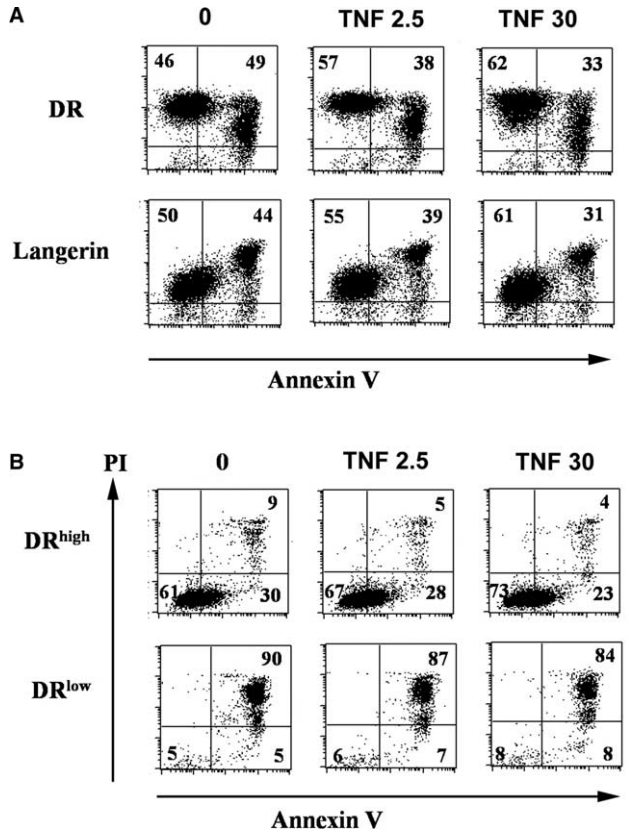

Fig. 4. TNF-α inhibits apoptosis of the HLA-DR^{high}/Langerin^{low} LC subset. (A) Fresh LC were seeded in serum-free medium in the absence (0) or presence of TNF-α at 2.5 (TNF 2.5) or 30 ng/ml (TNF 30) and analyzed for apoptosis after a 2-day culture. (A) Cells were double stained with PE-conjugated anti-HLA-DR or anti-langerin, and FITC-conjugated annexin V and analyzed without any gating. Numbers represent the percentage of LC in each quadrant. (B) LC apoptosis was assessed by double staining with annexin V-FITC and propidium iodide, and each of the two LC subsets were analyzed separately by gating them according to their size (FSC/SSC). In each dot plot, the percentage of LC in early apoptosis (Annexin V$^+$/PI$^-$: lower right quadrant), late apoptosis (Annexin V$^+$/PI$^+$ cells: upper right quadrant) and viable LC (Annexin V$^-$/IP$^-$: bottom left quadrant) is reported, and is representative of two separate experiments.

of IL-10, IL-6, IL-15, IL-1ß, TNF-α and no IP-10, IL-12 p40, IL-12 p70. When stimulated with TNF-α, IP-10 and IL-12 p40 were both induced. By contrast, the levels of IL-10, IL-6, IL-12 p70, IL-15 and IL-1ß did not change significantly compared with untreated LC. LC stimulation with CD40L-transfected cells plus IFN-γ resulted in high secretion of IP-10 and IL-12 p40, substantial quantity of IL-6 and IL-10, but few TNF-α and no IL-12 p70, IL-15 and IL-1ß (Table 2). Interestingly, a similar cytokine profile was obtained when LC were exposed to TNF-α, prior to or simultaneously with CD40L + IFN-γ (not shown).

LC stimulated with CD40L + IFN-γ exhibited similar typical mature morphology (e.g. numerous long dendrites) than those exposed to TNF-α (data not shown). This was confirmed by phenotypic analysis, showing that CD40L + IFN-γ enhanced the expression of most activation and maturation markers in a manner similar to that of TNF-α (not shown), thus demonstrating the efficiency of the stimulus. Although LC cultures are contaminated by minor numbers of keratinocytes, their influence seems rather marginal. Indeed, the absence of serum leads to a very low level of endogeneous cytokines, even when stimulated with CD40L + IFN-γ (Table 2), except for IP-10.

4. Discussion

Recent work has provided definite evidence for an important role of DC in immunity and tolerance, depending on their activation or maturation state. DC maturation is usually induced by ligands that bind to members of the TNF or toll-like receptor families. Here, we showed that TNF-α exposure of fresh immature LC, highly purified from healthy human skin, markedly increased the expression of co-stimulatory molecules (CD40, CD54, CD86), maturation markers (CD83, DC-LAMP) and the chemokine receptor CCR7 in a dose-dependent way. This mature phenotype is closely associated with enhanced LC allostimulating capacity, even at low doses of TNF-α (2.5 ng/ml). These results contrast with earlier studies in rodents, showing that TNF-α did not alter allostimulatory properties [9]. The discrepancy might be explained by species specificity or, more likely by the differences in experimental conditions, i.e. the LC purification yield, the presence or not of serum during LC cultures and the use of paraformaldehyde fixed cells in mixed lymphocyte cultures.

Migration of DC from peripheral tissues to lymphoid organs is a crucial step for their function. This is achieved through the up-regulation of CCR7, usually attributed to mature DC. We found that TNF-α strongly increased CCR7 surface expression on human LC, in a dose dependent way, indicating that TNF-α -activated LC may migrate into lymph nodes to prime T cells. Although our data are consistent with the well-known role of TNF-α in LC departure from epidermis [7], further studies are needed to determine whether responsiveness to CCR7 chemokine is functional.

After a 2-day culture, LC matured into two LC subsets, which could be distinguished by size, co-stimulatory molecules, maturation markers, and stage of apoptosis. Indeed, the small-sized/HLA-DR^{low}/Langerin^{high} LC subset likely represents a dying cell population, as assessed by annexin staining of more than 80% of the cells (Fig. 4). We showed that TNF-α increased the relative number and the maturation level of the large sized/HLA-DR^{high}/Langerin^{low} LC subset, a process partly due to the protective effect of TNF-α against spontaneous apoptosis. This is in agreement with previous data demonstrating that TNF-α maintains the viability of fresh murine LC [9], emigrated human LC [16], as well as in vitro generated DC [17].

While TNF-α -induced maturation is mediated exclusively via TNF-R1 and not TNF-R2 on in vitro generated DC [18], only TNF-R2 seemed to mediate TNF-α -effects on LC [19], probably due to the lack [20] or the very low [21] expression of TNF-R1. Consistent with this, we found that TNF-R2, but not TNF-R1, was expressed at the surface of both LC subsets, the highest intensity being expressed on the large sized/HLA-DR^{high}/Langerin^{low} LC subset. It is therefore conceivable that the differential ability of LC subsets to respond to maturational and anti-apoptotic TNF-α effects may result from quantitative differences in the cell surface expression of TNF-R2.

Table 2
TNF-α induces IP-10/CXCL-10 and IL-12 p40 secretion by human epidermal LC

	No stimulation	TNF-α	CD40L + IFN-γ
LC			
Expt 1			
IP-10	0	420	>5000
IL-12 p40	0	29.3	99.5
IL-10	3.2	3	60.4
IL-6	9.2	9.7	14.3
IL-12 p70	0	2.7	0
IL-8	>15 000	>15 000	>15 000
IL-15	1.7	1.3	4.7
IL-1SS	3.9	4.1	3.9
TNF-α	5	–	5.1
Expt 2			
IP-10	0	84	>5000
IL-12 p40	0	37	130
IL-10	1.8	2.7	9.7
IL-6	5.6	5.5	19.8
IL-12p70	0	0	1.2
IL-8	>15 000	>15 000	>15 000
IL-15	1	1.9	3
IL-1SS	4.2	3.9	3.9
TNF-α	5.4	–	7.5
Expt 3			
IP-10	0	459	>10 000
IL-12 p40	9.9	68.5	60.4
IL-10	5.5	6.2	6.8
IL-6	24	34	31
IL-12p70	2.7	5.4	2.1
IL-8	>15 000	>15 000	>15 000
IL-15	2	2.7	2.9
IL-1SS	5.6	5.6	5.7
TNF-α	5.1	–	5.3
Keratinocytes			
Expt 1			
IP-10	0	37	4355
IL-12 p40	7.9	0	7.9
IL-10	5.4	0	5.4
IL-6	0	0	22.8
IL-12 p70	0	0	1.2
IL-8	1204	3521	2375
IL-15	1.9	1.8	1.8
IL-1SS	5.6	5.6	5.6
TNF-α	5.1	–	5.6
Expt 2			
IP-10	0	49.7	n.d.
IL-12 p40	7.9	12.1	n.d.
IL-10	5.4	5.4	n.d.
IL-6	7.4	13.2	n.d.
IL-12p70	0	0	n.d.
IL-8	2530	8886	n.d.
IL-15	1.8	1.8	n.d..
IL-1SS	5.6	5.6	n.d.
TNF-α	5	–	4.9
Expt 3			
IP-10	0	55.2	n.d.
IL-12 p40	0	7.9	n.d.
IL-10	5.4	5.4	n.d.
IL-6	8	11.7	n.d.
IL-12p70	0	0	n.d.
IL-8	2852	10 926	n.d.
IL-15	1.8	1.8	n.d.
IL-1SS	5.6	5.7	n.d.
TNF-α	3.8	–	3.6

Highly purified fresh LC were cultured with TNF-α (30 ng/ml) or CD40 L-transfected cells combined with of IFN-γ (500 U/ml). Pellets of lymphoprep, which consisted mainly of keratinocytes were cultured in the same way as LC. After 2 days, supernatants were collected and cytokine concentration was measured by cytometric bead array assays or ELISA. Results are expressed as pg/ml. n.d.: not determined.

The ability of DC to secrete inflammatory cytokines and chemokines is of great importance to amplify and direct the type of immune response as well as to recruit host immune effectors [5,6,22,23]. Generally, secretion of the immunostimulatory form of IL-12, IL-12 p70 heterodimer consisting of p35 and p40 subunits, is required to induce Th1 responses, whereas production of IL-10 favors Th2 activation and in some cases regulatory T cell generation. Some groups have reported that human LC emigrating from skin explants did not make measurable amounts of bioactive IL-12 p70, even after stimulation with CD40L ± IFN-γ [24,25]. In these situations, LC were mature at the time of CD40 ligation, which might explain the lack of cytokine production. Indeed, DC matured by various stimuli were unable to produce IL-12 p70 upon subsequent CD40 ligation [22]. Here, we showed for the first time that TNF-α exposure of fresh immature LC induced IL-12 p40, but not IL-12 p70 secretion, as also reported for in vitro generated DC [26]. However, whereas CD40 ligation combined with T cell-derived co-signals, such as IFN-γ, triggered full maturation and optimal IL-12 p70 production by monocyte-derived DC [15], it did not induce human LC to secrete IL-12 p70, but only its p40 subunit. It is noteworthy that LC generated in vitro from $CD34^+$ progenitors are also unable to produce IL-12 p70 after stimulation with CD40L [27], in contrast to LC generated from monocytes [28].

The failure of human epidermal LC to produce IL-12 p70 has also been observed in response to peptidoglycan, flagellin and LPS [29]. In agreement with this, we found that strong IL-12 p70 inducers, including bacterial CpG DNA, viral poly IC, LPS and combination of CD40L with IL-4, were quite all unable to induce detectable amounts of Il-12 p70, even after further ligation with CD40L-transfected cells combined with IFN-γ (manuscript in preparation). The lack of IL-12 p70 could not be related to the assay procedure, since, using the same method and half lower cell concentration, substantial amount of IL-12 p70 were found after CD40 cross-linking of monocyte-derived DC [30]. Furthermore, IL-10, known to inhibit bioactive IL-12 production by DC [31] was undetectable. Given that IL-12 p40 antagonized IL-12 p70 bioactivity and thus may act in an anti-inflammatory manner [23], it is tempting to speculate that LC themselves can limit the progression of potentially devastating inflammation within the skin.

An important finding is that TNF-α induces LC to secrete significant levels of IFN-γ inducible protein 10 (IP-10/CXCL10), a chemokine responsible for skin homing of Th1 type lymphocytes [32]. Keratinocytes, which slightly contaminate the highly purified LC, can also produce IP-10 under TNF-α treatment [33], but in our assays, the data implicate LC as the main source of the chemokine (Table 2). This could be related to the serum-free conditions, which was shown to induce a decrease of keratinocyte-derived soluble mediators [34]. IP-10 is largely produced in chronic inflammatory diseases such as psoriasis [35] and the present results suggest that, in addition to keratinocytes, LC might be involved in the chemokine production. Recently, Fujita et al. [36] reported that exogeneous IL-12 could induce IP-10 production by murine LC. Whether endogeneous IL-12 p40 induced by human LC under exposure to TNF-α might be responsible for IP-10 secretion remains to be defined. Although the question has not been addressed here, it would be also of interest to determine the outcome of T cell response.

In conclusion, our data provide novel information regarding the effect of TNF-α on human epidermal LC. TNF-α favours the semi-maturation of LC, characterized by phenotypic maturation but lack of IL-12 p70 production. Furthermore, through the induction of both IL-12 p40 and IP-10 production, TNF-α may confer to human epidermal LC a dual role in up- or down-regulating the inflammatory skin responses.

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